Supplementary Information

Methods

Cell cultures

Primary microglia were established from P1 Sprague Dawley rats as previously described (32). Primary neuronal cultures were obtained from the hippocampi of 18-day-old fetal Sprague Dawley rats (Charles River Italia), as reported (32). Briefly, dissociated cells were plated onto poly-L-lysine (Sigma Aldrich) treated coverslips, and were maintained in Neurobasal with 2% B27 supplement (Invitrogen).

Isolation of MVs, exosomes or mixed EVs

Microglia was stimulated with 1 mM ATP for 1 h in Krebs-Ringer's HEPES solution (KRH) (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO, 2 mM $CaCl_2$, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4). Conditioned KRH was collected and pre-cleared from cells and debris at 300g for 10 min (twice). Quite large MVs were then pelleted from the supernatant by a centrifugation step at 10,000*g* for 30 min, while smaller exosomes were subsequently pelletted at higher speed, i.e. 100,000g for 1h. To collect mixed EVs, KRH pre-cleared from the 300g pellet, was directly centrifugated at 100,000g. Vesicle pellets were resuspended and used immediately after isolation for Nanosight analysis, fluorescent microscopy and electrophysiological recordings, resuspended in SDS sample buffer for western blotting, or stored at -80°C for AEA and 2-AG determination. Protein content of EVs and donor cells was determined by Micro BCA protein assay kit and BCA respectively (Thermo Fischer Scientific).

Nanosight analysis

The number and dimension of MVs and exosomes was assessed with Nanosight LM10-HS system configured with a 405 nm laser and EMCDD camera (Hamamatsu Photonics). Videos were

collected and analysed using the NTA-software (version 2.3), with the minimal expected particle size, minimum track length, and blur setting, all set to automatic. Camera shutter speed was fixed at 20.01 ms and camera gain was set to 350. Ambient temperature was ranging from 25 to 28°C. MV- and exosome-enriched pellets were resuspended to 400µl of 0,1 µm-filtered sterile KRH and five recordings of 30 seconds were performed for each sample. No particles were detected in 0,1 µm-filtered sterile KRH.

Drugs and EV treatments

For electrophysiological experiments, neurons were acutely exposed to the CB_1 receptor agonist WIN55,212-2 (1 µM, Sigma-Aldrich; previously diluted with saline (0.9% NaCl) and Tween-80 (2%)) or pre-incubated for 15 min with the CB_1 receptor antagonist SR141716A (1 µM, Tocris) and then exposed to MVs (1.2 µg/ml, produced by microglia in a microglia-to-neuron ratio of 2:1) in the presence of the antagonist for 40-45 min. OEA treatments (37 µM, Sigma-Aldrich) were performed according to (32). MVs were broken by ipo-osmotic stress (via suspension in 7.3 mM Phosphate Buffer at 4°C for 30 min) and re-pelletted at 100,000*g* for 1h. To block MV shedding, oATP (100 µM) was pre-administrated to microglia for 10 min and then co-administrated together with ATP (1 mM) for 30 min.

For biochemical experiments neurons, untreated or pre-incubated for 15 min with the CB_1 receptor antagonist, were exposed to MVs (7 µg/ml, produced by microglia in a microglia-to-neuron ratio of 10:1) for different time periods at 37°C and were processed for protein extraction as described below.

Western blotting

Total cellular or vesicular extracts were separated by electrophoresis, blotted on nitrocellulose membrane and probed using rabbit anti-ERK 1/2 and anti-P-ERK 1/2 T202/Y204 (1:1000; Cell Signalling), mouse anti-Tsg101 (1:500; Abcam), rabbit anti-alix (1:500; Covalab), mouse anti-

flotillin (1:1000; BD Transduction), mouse anti-SP1 (1:5000; Upstate), rabbit anti-TOM-20 (1:500; Santa Cruz Biotechnology), rabbit anti-adypophilin (1:200; Santa Cruz Biotechnology), **mouse anti-GS28 (1:1000; BD Transduction)**. Photographic development was by chemiluminescence (ECL, GE Healthcare or Immobilon substrate, Millipore), according to the manufacturer's instructions. Western blot bands were quantified by ImageJ software.

Endogenous levels of AEA and 2-AG

Lipids were extracted from EVs or donor cells ($50x10^6$ N9 cells/$15x10^6$ microglia) with chloroform/methanol (2:1, v/v), in the presence of d_8-AEA and d_8-2-AG as internal standards. The organic phase was dried, and then was analyzed by LC-ESI-MS, with a Perkin Elmer LC system (Perkin Elmer, Waltham, MA, USA) in conjunction with a single quadrupole API-150EX mass spectrometer (Applied Biosystems, Foster City, CA, USA). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as previously reported (47).

Immunofluorescence staining

Immunofluorescence staining of fixed neurons was performed using the following antibodies: rabbit anti-CB_1 (1:500; Synaptic System), guinea pig anti-vGLUT1 (1:1000; Synaptic System), human anti-GAD (kindly gift of Prof M. Solimena, Medical School, TU Dresden), mouse anti-SNAP-25 (SMI 81 1:500; Sternberger). Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen). Goat anti-GFP FITC-conjugated antibody (Novus Bioogical) was used to reveal farnesyl-GFP-expressing ectosomes, produced by glial cultures transfected with pEGFP-F Vector (BD Biosciences Clontech). Images were acquired using a Leica TCS SPE confocal microscope. Fluorescence images were processed with ImageJ.

To visualize AEA, MVs produced by microglia preloaded with calcein-AM were incubated with 5 μM biotin-AEA or maintained in 1ml KRH (control) for 10 min at 37°C (Tocris). After dilution in KRH (12 ml), MVs were pelleted at 10.000g, and re-suspended in KRH (50 μl) containing Cy3-

streptavidine (1:200) for 30 min. After dilution in KRH (12 ml), MVs were finally re-pelletted, resuspended in 100 µl of KRH, spotted on glass coverslip and observed with a Leica TCS SP5 confocal microscope.

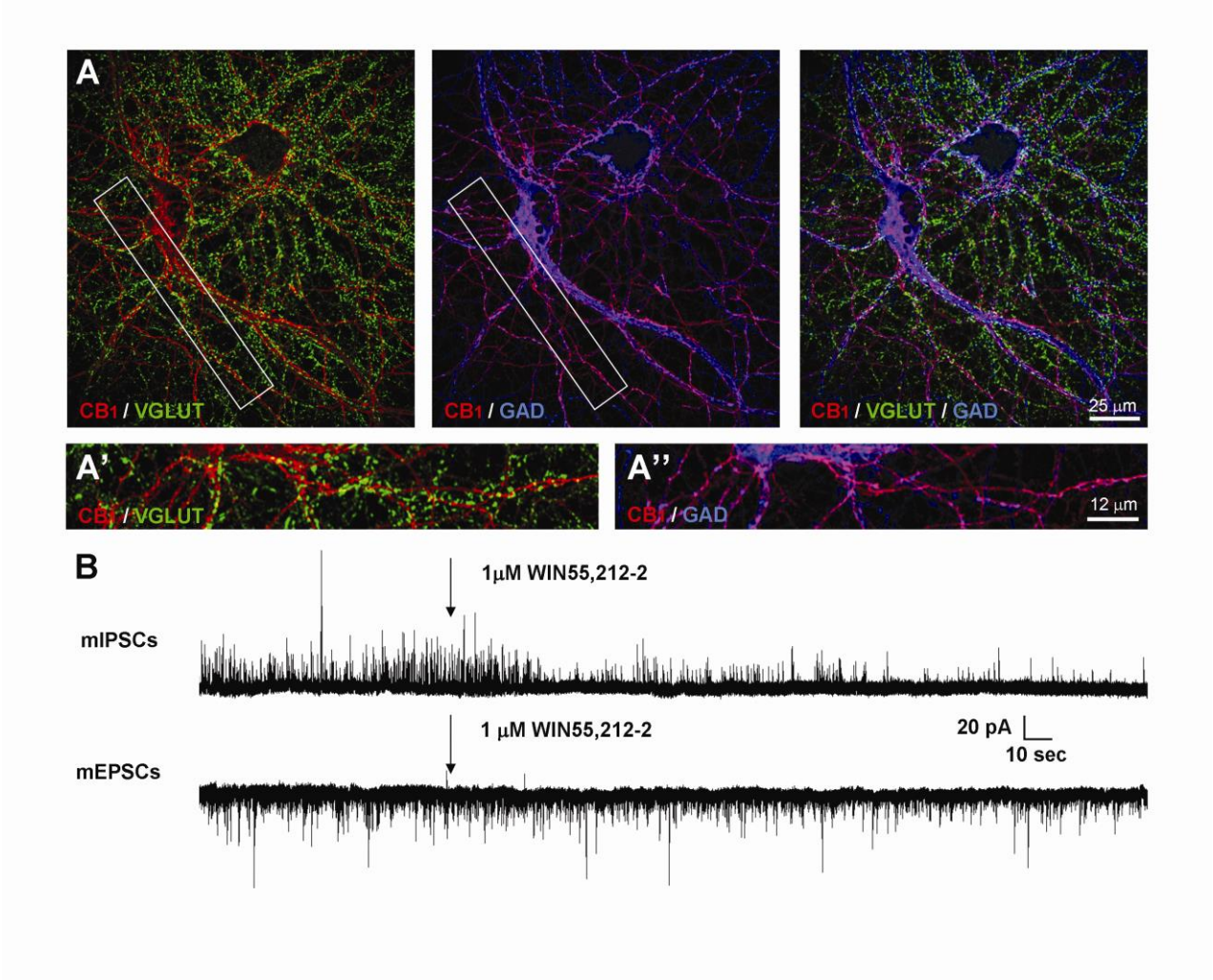
Electrophysiological recordings

Whole-cell voltage clamp recordings were performed using a MultiClamp 700A amplifier (Axon Instruments) coupled to a pCLAMP 10 Software (Molecular Devices), and using an inverted Axiovert 200 microscope (Zeiss). Experiments were performed at room temperature (20–25°C) in the external control solution KRH. Signals were sampled at 10 kHz and filtered to 4 kHz. mIPSCs were recorded from 16-20 DIV hippocampal neurons, with an holding potential of +10 mV in the presence of 1 µM tetrodotoxin (TTX, Tocris), using the following internal solution: 130 mM CsGluc, 8 mM CsCl, 2 mM NaCl, 10 mM HEPES, 4 mM EGTA, 4 mM MgATP, 0,3 mM Tris-GTP (pH 7.3, adjusted with CsOH). mIPSC traces were analyzed with Clampfit Software using a threshold of 5 pA. The mean mIPSC frequency was about 1,4 Hz, the mean amplitude about 14 pA. mEPSC were recorded from 13-15 DIV neurons, with an holding potential of -70 mV and in the presence of 1 µM TTX as previously described (32). mEPSC traces were analysed with MiniAnalysis Program (Synaptosoft) using a threshold of 10 pA. The mean mEPSCs frequency was about 1,4 Hz.

Statistical analysis

All data are presented as mean±SE from the indicated number of experiments. Statistical analysis was performed using SigmaStat 3.5 (Jandel Scientific, San Jose, CA, USA) software. After testing data for normal distribution, the appropriate statistical test has been used (see figure legends). Differences were considered significant when P was <0.05, indicated by an asterisk; those at P was <0.01 are indicated by double asterisks and those at P <0.001 are indicated by triple asterisks.

Supplementary Figure



Supplementary Figure 1 Expression and function of CB_1 in hippocampal neurons *in vitro*

(A) Immunofluorescence of 17 DIV old primary rat hippocampal neurons, showing an almost exclusive localization of CB_1 on GABAergic terminals. Cells were stained for CB_1 (in red), for the vesicular glutamate transporter VGLUT (in green), a marker of glutamatergic neurons and for glutamic acid decarboxylase GAD (in blue), a neuronal marker expressed in GABAergic neurons. Scale bar corresponds to 25 µm. A' and A" insets show details of CB_1/VGLUT and CB_1/GAD labeling at higher magnification. Scale bar corresponds to 12 µm. (B) Representative traces of mIPSCs and mEPSCs from neurons exposed to 1 µM WIN55,212-2.